MOLECULAR IDENTIFICATION

Development of a PCR assay specific for Peptostreptococcus anaerobius

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Peptostreptococcus anaerobius is a gram-positive anaerobic coccus that is widely distributed in the normal human flora. The organism has also been implicated as a causative agent of several systemic infections, including endocarditis and infections of the genitourinary and gastrointestinal tracts. Its role in oral disease is less well defined, although it has been implicated in periodontal disease, gingivitis and root canal infections. Identification of P. anaerobius in clinical samples is currently reliant upon traditional culture and biochemical methods. The aim of this study was to develop a novel PCR assay for the detection of P. anaerobius and to attempt detection of this organism in oral samples. PCR primers specific for P. anaerobius DNA were developed by alignment of bacterial 16S ribosomal RNA gene sequences and selection of sequences specific at their 3’ ends for P. anaerobius. When used in a PCR assay, positivity for P. anaerobius DNA was indicated by the amplification of a 943-bp product. The primers were shown to be specific for P. anaerobius DNA, as no PCR products were obtained when genomic DNA from a wide range of other Peptostreptococcus species and other oral bacteria were used as templates. The PCR assay was then applied to the detection of P. anaerobius DNA in subgingival plaque samples from adult periodontitis patients and pus aspirates from subjects with acute dento-alveolar abscesses. All of 60 subgingival plaque samples from 16 patients were negative for P. anaerobius DNA. None of the 43 pus samples analysed contained P. anaerobius DNA. These results suggest that P. anaerobius is not a major pathogen in adult periodontitis and dento-alveolar abscesses. The PCR assay is a more rapid, sensitive and specific alternative to culture-based methods for identification of P. anaerobius in clinical samples.

Introduction

Peptostreptococcus anaerobius is a gram-positive anaerobic coccus that is the type species of the genus Peptostreptococcus [1]. P. anaerobius is regarded as a member of the normal gastrointestinal flora, although it is uncertain as to whether it is a member of the normal oral and vaginal flora [2]. The organism is involved in polymicrobial infections and has been isolated from a wide variety of human clinical specimens. These include abscesses of the brain, ear, jaw, pleural cavity, pelvic, urogenital and abdominal regions [1], nasal septal abscesses [3], infected haemorrhoids [4], infections of the abdominal cavity [5] and of the female genitourinary tract [6]. P. anaerobius is also involved in soft tissue infections, including those of the leg and external genitalia [7]. The isolation of P. anaerobius in pure culture from a case of endocarditis has been reported [8].

The involvement of P. anaerobius in oral infections such as periodontal disease is less well defined. P. anaerobius has been associated with gingivitis and periodontitis [9, 10], although an earlier study suggested that the organism was found in subgingival plaque at very low levels [11]. P. anaerobius is one of the species found most frequently in the root canals of teeth with periapical periodontitis [12] and has been isolated from a peritonsillar abscess [13].

Identification of P. anaerobius in clinical samples has traditionally been accomplished by a combination of microbiological culture and biochemical tests. These tests are based upon carbohydrate fermentation reactions and production of saccharolytic and proteolytic
enzymes, and have been used with limited success in identifying P. anaerobius [2, 14, 15]. Volatile fatty acid (VFA) production has proved to be a useful identification method, as P. anaerobius is the only gram-positive anaerobic coccus to produce isocaproic acid as the major end-product of metabolism [2].

Although some of these traditional methods have proved useful in the identification of P. anaerobius, their routine use in a diagnostic laboratory is often hindered by the ambiguous nature of their results and by the fact that they are laborious and expensive to carry out. The purpose of this study was to develop a novel PCR assay for the specific detection of P. anaerobius DNA in clinical samples, that could be used as a more reliable, specific and rapid alternative to currently used detection methods. The present report describes the development of a PCR assay specific for P. anaerobius DNA and its application in the attempted detection of P. anaerobius in subgingival plaque samples from adult periodontitis patients and pus aspirates from subjects with acute dento-alveolar abscesses.

Materials and methods

Bacterial culture and genomic DNA extraction

P. anaerobius ATCC 27337 was cultured on Fastidious Anaerobe Agar (Life Technologies, Paisley) supplemented with defibrinated horse blood 7.5% v/v and incubated at 37°C for 4–5 days in an anaerobic chamber in an atmosphere of N₂ 85%, CO₂ 10% and H₂ 5%. Bacteria were harvested from the plates and genomic DNA was extracted with the Puregene DNA Isolation Kit (Novara Flowgen, Ashby de la Zouch).

Sample details

Subgingival plaque was obtained from patients with untreated chronic inflammatory adult periodontitis who had been newly referred to Glasgow Dental Hospital. Criteria for inclusion in the study were the presence of at least three periodontal pockets with a depth of at least 5 mm and bleeding on probing, together with no history of antibiotic treatment during the preceding 6 months. In total, 60 subgingival plaque samples from 16 patients were analysed. The mean pocket depth of analysed samples was 7.2 mm (range 5–10 mm); the age range of the patients was 29–58 years (mean age 42.1 years). One pus sample from each of 16 subjects with acute dento-alveolar abscess was analysed; their age range was 32–54 years (mean 40.8 years).

Sample collection and preparation

Subgingival plaque samples were collected with a sterile curette into sterile tubes containing 0.5 ml of freshly prepared Fastidious Anaerobe Broth (Bioconnections, Leeds). Samples were mixed for 30 s and lysates were prepared by adding 3 μl of achromopeptidase (20 U/μl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.0) to 100 μl of plaque. Samples were incubated at 56°C for 30 min, boiled for 5 min and stored at −70°C until required.

A 50-μl volume of each pus sample was diluted 10–100-fold in PCR diluent (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA); 30 μl of SDS 10% and 3 μl of proteinase K (10 mg/ml) were added to 300 μl of diluted pus and incubated at 55°C for 3 h. Lyed samples were extracted twice with an equal volume of phenol:chloroform (1:1) and once with an equal volume of chloroform. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.3, and 2 volumes of ethanol 100%, followed by mixing and incubation at −70°C for 30 min. Precipitated DNA was recovered by centrifugation and the pellet was resuspended in 100 μl of sterile molecular biology grade water.

Selection of PCR primers

Primers for use in the PCR assay were derived from the 16S rRNA gene sequence of P. anaerobius ATCC 27337. The 16S rRNA gene sequences of Peptostreptococcus species and several other oral bacteria were aligned with Version 8 of the University of Wisconsin Genetics Computer Group sequence analysis program package [16]. Two primers that demonstrated sequence specificity for P. anaerobius at their 3’ ends were selected from unique regions of the P. anaerobius 16S rRNA gene sequence. The primer sequences were: 5’-CGT CTW ATT TNA GC AAG GGG TCT-3’ (Pan-1; base position 62–81), where N = A + C + G + T and W = A + T, and 5’-AGC CCC GAA GGG AAG GTG TG-3’ (Pan-2; base position 1004–985), which give an expected amplification product of 943 bp.

PCR

All PCR reactions were performed in a total volume of 50 μl and the PCR reaction mixture was essentially as described previously [17]. Each PCR reaction mixture comprised either 5 μl of lysed plaque sample/pus extract or 1 μl of bacterial genomic DNA and either 45 or 49 μl of reaction mixture containing MgCl₂ at the optimum concentration of 1.5 mM. The PCR cycling conditions comprised an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and extension at 72°C for 1.5 min, and finally an extension step at 72°C for 10 min.

Stringent anti-contamination procedures were employed when performing PCR, as described previously [17]. Negative and positive controls were included with each batch of samples being analysed. The positive control was a standard PCR reaction mixture containing 10 ng of P. anaerobius genomic DNA instead of sample; the
negative control contained sterile water instead of sample. PCR products were visualised by electrophoresis on agarose 2% gels as described previously [17].

**Bacterial species used as PCR controls**


**Results**

**Specificity and sensitivity of the *P. anaerobius* PCR assay**

After 35 cycles of amplification, the lower limit of detection of *P. anaerobius* was c. 50 bacterial cells (data not shown). The specificity of the PCR assay was confirmed by performing PCR with 10 ng of genomic DNA from each of the bacterial species selected for use as controls. Only the use of *P. anaerobius* DNA as a template resulted in a PCR product of the expected size (Fig. 1, lane 2).

**PCR analysis of clinical specimens**

The *P. anaerobius* PCR assay was used to determine the prevalence of this species in subgingival plaque samples from patients with adult periodontal disease and in pus aspirates obtained from subjects with acute dento-alveolar abscesses. *P. anaerobius* DNA was not detected by PCR in any of 60 subgingival plaques and 43 pus samples analysed. Sample inhibition of PCR was discounted because the spiking of selected plaque

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![Fig. 1. Specificity of the *P. anaerobius* PCR assay. Electrophoresis on an agarose 2% gel of PCR products obtained with primer pair Pan-1 and Pan-2 and genomic DNA from various bacteria as template: *P. magnus* NCTC 11804 (lane 1); *P. anaerobius* ATCC 27337 (2); *P. micros* ATCC 33270 (3); *P. asaccharolyticus* NCTC 11461 (4); *P. prevotii* NCTC 11806 (5); *P. tetradius* ATCC 35098 (6); *P. productus* NCTC 11829 (7); *P. indolicus* NCTC 11088 (8); *P. intermedia* ATCC 25611 (9); *P. gingivalis* ATCC 33277 (10); *B. forsythus* ATCC 43037 (11); *H. pylori* ATCC 43504 (12); *A. actinomycetemcomitans* ATCC 33384 (13); *F. nucleatum* ATCC 25586 (14); *Eik. corrodens* ATCC 23834 (15); *S. mutans* ATCC 25175 (16); *S. aureus* ATCC 12600 (17); *A. naeslundii* ATCC 12104 (18); 100-bp DNA ladder (19); negative control (20). PCR positivity is indicated by the presence of a 943-bp product.**
and pus samples with 1000 whole cells of *P. anaerobius* yielded a PCR product of the expected size (data not shown).

**Discussion**

The aims of this study were to develop a novel PCR assay specific for *P. anaerobius* and to use this assay to determine the incidence of this organism in oral specimens. Conventional methods for identification of *P. anaerobius* and other members of the Peptostreptococcus genus are reliant upon bacterial culture coupled with biochemical identification of clinical isolates. *P. anaerobius* is commonly identified on the basis of colony morphology and Gram’s staining characteristics, followed by biochemical analyses. The Rapid ID 32A system, which analyses bacterial sugar fermentation characteristics and enzymatic activities, has been used for identification of *P. anaerobius* [18]. This method is perhaps of more value for identification of *P. anaerobius* than other members of the Peptostreptococcus genus, because *P. anaerobius* is more saccharolytic than other species in this genus. The RapID ANA II panel of tests identifies bacteria on the basis of their capacity to hydrolyse amino acid and phosphate substrates. However, this method has proved to be unreliable for identification of *P. anaerobius*, as in one study 37% of *P. anaerobius* isolates were misidentified [14]. Detection of VFA end-products of metabolism by gas-liquid chromatography has also been used to identify *Peptostreptococcus* spp. [2, 15, 19]. This has proved to be a reliable method for identification of *P. anaerobius*, as it is the only Peptostreptococcus species to produce a major terminal peak of isocaproic acid.

These conventional methods for identification of *P. anaerobius* have undoubtedly been of considerable use. However, because of the emergence of phenotypically variable strains with altered biochemical characteristics, ambiguous results are often obtained with such methods. Other considerations are the lengthy processes involved and the high overall costs of performing such tests. PCR offers an alternative method of identification that is simpler, cheaper and quicker than conventional methods. Furthermore, as PCR can detect phenotypically variable strains unequivocally, it can be considered as a more specific and reliable method for bacterial identification.

In the present study, a PCR assay for the specific detection of *P. anaerobius* was developed and evaluated. The assay was shown to be specific for *P. anaerobius*, as no other bacterial species tested – which included the most closely related *Peptostreptococcus* spp. – were detected with the PCR primers used. When the PCR assay was used to attempt detection of *P. anaerobius* in subgingival plaque samples from adult periodontitis patients and pus aspirates from dento-alveolar abscesses, no clinical specimens examined were found to harbour the organism. This is in contrast to previous findings for *P. micros*, which was detected in 19 (28%) of 60 subgingival plaque samples and 20 (71%) of 28 pus samples analysed by PCR [20]. This is not an unexpected finding, because the role of *P. anaerobius* in oral infections is less well defined than that of *P. micros*, which has been well documented as an oral pathogen [2]. Very few previous studies have attempted to detect *P. anaerobius* in oral specimens. The tenuous nature of the link between *P. anaerobius* and periodontal disease is further documented by the immunological findings of another study. With a gingival explant culture system, Hall *et al.* [21] evaluated the reactivity of local antibodies produced by juvenile periodontitis tissue. Only 2 of 73 gingival explant culture supernatant fluids demonstrated reactivity to *P. anaerobius*, indicating that this bacterium does not play a major role in periodontal disease.

No clinical samples were shown to be PCR-positive for *P. anaerobius* in the present study. However, confirmation of PCR product identity in any future studies could readily be done by digestion of PCR products with a combination of the restriction endonucleases Rsal, *Mnl* and *Hinfl*, which give a distinct restriction profile for *P. anaerobius* DNA.

The classification of members of the genus *Peptostreptococcus* has generated much discussion. 16S rRNA sequence analysis has demonstrated that *P. anaerobius* is distinct from other anaerobic gram-positive micro-organisms [22, 23]. Several studies have suggested that other members of the genus *Peptostreptococcus* should be removed from this genus and reclassified. On the basis of 16S rRNA sequence data, it has been proposed that *P. productus* be placed in the genus *Ruminococcus* [24]. It has also been proposed that, on the basis of biochemical analysis and 16S rRNA sequence data, *P. magnus* be reclassified in the new genus *Finegoldia* as *Fin. magna*, and that *P. micros* be reclassified in the new genus *Micromonas* as *M. micros* [25]. It was recently proposed that *P. anaerobius* should be the only remaining member of the genus *Peptostreptococcus* [26]. In that study, phylogenetic analysis of 16S rRNA sequence data demonstrated that *Eubacterium brachy* and *Clostridium mayombei* were most closely related to *P. anaerobius*, although it was advocated that these species should not be placed in the same genus as *P. anaerobius*. The primers specific for *P. anaerobius* used in the present study are very divergent in sequence at their 3’ ends from the corresponding region of the 16S rRNA genes of *E. brachy* and *C. mayombei*, and as such would not be capable of detecting these closely related species.

In conclusion, a novel PCR assay for the specific detection of *P. anaerobius* has been developed. This PCR assay was used to assess the prevalence of *P. anaerobius* in subgingival plaque from adult perio-
References


